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Identification and characterization of multiple conserved nuclear localization signals within adenovirus E1A

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ABSTRACT

The human adenovirus 5 (HAdV-5) E1A protein has a well defined canonical nuclear localization signal (NLS) located at its C-terminus. We used a genetic assay in the yeast *Saccharomyces cerevisiae* to demonstrate that the canonical NLS is present and functional in the E1A proteins of each of the six HAdV species. This assay also detects a previously described non-canonical NLS within conserved region 3 and a novel active NLS within the N-terminal/conserved region 1 portion of HAdV-5 E1A. These activities were also present in the E1A proteins of each of the other five HAdV species. These results demonstrate that, despite substantial differences in primary sequence, HAdV E1A proteins are remarkably consistent in that they contain one canonical and two non-canonical NLSs. By utilizing independent mechanisms, these multiple NLSs ensure nuclear localization of E1A in the infected cell.

Introduction

The defining characteristic of eukaryotic cells is the separation of the intracellular space into membrane bound compartments, including the nucleus where nucleic acid synthesis and processing occurs. Transport systems exist that allow proteins to be imported into the nucleus from the cytoplasm, as well as the export of proteins and RNA to the cytoplasm. Nuclear import of many cellular and viral proteins is typically mediated by nuclear localization signals (NLS) that physically interact with soluble cytosolic receptor proteins (Macara, 2001). A canonical monopartite NLS contains a short single stretch of at least three basic amino acids (B) with a consensus sequence fitting B_4, P(B_3X), PXX(B_3X) or B_3(H/P), where P is proline, H is histidine, X is any amino acid and letters in parentheses can be in any order (Macara, 2001; Nakai and Horton, 1999). Alternatively, a canonical bipartite NLS contain two short stretches of basic amino acids separated by a non-conserved sequence (Macara, 2001).

Proteins containing canonical NLSs interact in the cytosol with the importin α family of NLS receptors (also known as karyopherin α). Subsequent heterodimerization of importin α with importin β (also known as karyopherin β) and interaction with components of the nuclear pore complex leads to translocation into the nucleus in a GTP dependent fashion (Macara, 2001). The yeast *Saccharomyces cerevisiae* expresses only one importin α, Srp1 (Enenkel et al., 1995). In contrast, there are multiple mammalian importin α proteins, which are divided into three subtypes, represented by Rch1 (human importin α1), Qip1 (human importin α3) and NPI1 (human importin α5) (Miyamoto et al., 1997). The total amount of importin α, as well as the relative content of each importin α subtype, varies among different cell types and during different stages of development (Poon and Jans, 2005). Each importin α isoform has distinct substrate specificities, which may confer a level of regulation to nuclear import (Macara, 2001). Although many thousands of cellular proteins are transported into the nucleus, very few NLSs have been characterized in detail. Existing work has been heavily biased towards canonical monopartite or bipartite signals. Recently, NLSs that are not particularly rich in basic amino acid residues have been identified that also interact with importin α. As these import signals do not

Abbreviations: HAdV, human adenovirus; NLS, nuclear localization signal; E1A, early region 1A; R, residue; DBD, DNA binding domain; AD, activation domain; CR, conserved region; MBP, maltose binding protein; ITT, inducible translocation trap

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conform to either the monopartite or bipartite types described above, they are referred to as non-canonical. Many of these signals have been identified in viral proteins, such as the influenza virus NP protein, the Borna disease virus p10 protein, cytomegalovirus UL84 protein and the Varicella-Zoster virus ORF29 protein (Wolff et al., 2002; Lischka et al., 2003; Stallings and Silverstein, 2005; Cros et al., 2005).

There are over 50 types of human adenovirus (HAdV), divided into six species. HAdV-5, a species C virus, is the most thoroughly characterized. The first gene expressed during HAdV-5 infection is Early Region 1A (E1A). The largest E1A protein contains 289 amino acid residues (289R) and has four highly conserved regions (CR1-4; Fig. 1A) (Avvakumov et al., 2004). HAdV-5 E1A is localized to the nucleus and contains a well characterized monopartite NLS (KRPRP) located at its C-terminus (Lyons et al., 1987). This NLS mediates nuclear import *in vitro* and *in vivo*, shows a distinct preference for human Qip1 (importin α3) *in vitro* (Kohler et al., 2001), and is regulated by acetylation (Madison et al., 2002). However, not all the E1A proteins of the different types, specifically those encoded by species B viruses, contain this predicted classical NLS (Avvakumov et al., 2004). A second non-canonical NLS, with the consensus sequence $FV(X)_{7\text{-}26}MXSLXYM(X)_4MF$, spans residues 142–182 in CR3 of HAdV-5 E1A, and this sequence is unique to the species C E1A proteins (Slavicek et al., 1989; Standiford and Richter, 1992). This sequence does not resemble other known NLSs and when expressed in *Xenopus* embryos functions only during early neuronal stage, suggesting that it may be developmentally regulated (Standiford and Richter, 1992). At least one other non-canonical NLS may be present within the HAdV-5 E1A protein, as residues 23–120 have been reported to be sufficient to mediate nuclear accumulation in micro-injected *Xenopus laevis* oocytes (Richter et al., 1985).

We have utilized a simple and sensitive genetic method to identify and characterize novel NLSs in E1A. This system is based on the expression of the test protein fused to a modified LexA DNA binding domain (DBD) and the Gal4 transcriptional activation domain (AD) in the yeast *S. cerevisiae* (Marshall et al., 2007). The test protein is too large to passively diffuse into the nucleus and only when fused to a functional NLS will the chimera enter the nucleus and activate transcription of a LexA responsive β-galactosidase reporter gene. Furthermore, and the system is not limited to the identification of yeast NLSs, as the nuclear import apparatus is highly conserved between yeast and higher eukaryotic cells (Macara, 2001; Rhee et al., 2000).

Our previous work has shown that the yeast transcription based assay detects the canonical C-terminal NLS, the non-canonical NLS in CR3 and an N-terminal activity in HAdV-5 E1A (Marshall et al., 2007). In this study, we demonstrate the E1A proteins of all six HAdV species contain a functional canonical NLS at their C-terminus, a non-canonical NLS within CR3 and a third non-canonical NLS within the N-terminal/CR1 region. We also show that the N-terminal/CR1 NLS interacts indirectly with importin α and functions in mammalian nuclear import assays. Thus, despite substantial differences in primary sequence, HAdV E1A proteins are remarkably consistent in that they contain multiple NLSs. This redundancy may ensure nuclear localization of the viral protein during infection regardless of the importin α expression profile.

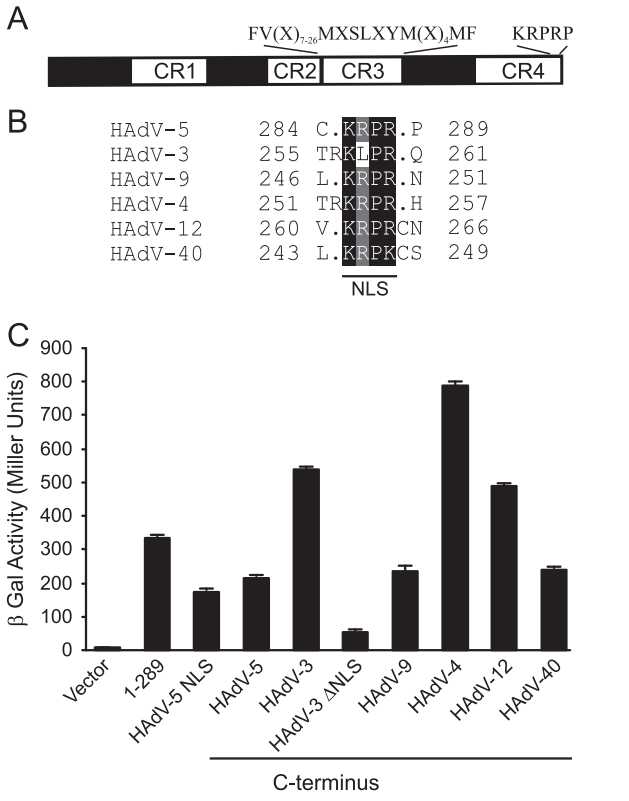

Fig. 1. Map of E1A and analysis of nuclear import mediated by the C-terminus of E1A from representative HAdV types. (A) Map of the 289R E1A protein showing the location of the conserved regions (CR1-4) and known nuclear localization signals. (B) Alignment of the C-terminal E1A NLSs from representative HAdV types used in this study (Avvakumov et al., 2004). (C) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for the C-terminal E1A regions from the indicated HAdV types (means ± SD, $n=3$). Vector—pNIA-CEN-MBP.

Results

The canonical NLS located at the C-terminus of E1A is functional in all HAdV species

We have previously described an improved transcription based assay in yeast for identifying signals that direct nuclear import of a fusion protein that exceeds the diffusion limit of the nuclear pore (Marshall et al., 2007). In this assay, fusion of a functional NLS to the large cytoplasmically localized transcription factor induces the chimera to enter the nucleus and activate transcription of a LexA responsive β-galactosidase reporter gene. This system detects the canonical C-terminal NLS in HAdV-5 E1A (Marshall et al., 2007). Although the E1A proteins from all HAdV species contain a short stretch of basic amino acid residues near their C-terminus, the E1A proteins from many species B viruses, including HAdV-3, do not have a predicted canonical NLS (Fig. 1B). To determine if each of these regions could direct nuclear import, we tested the C-terminal portions of each of the HAdV-3, 4, 5, 9, 12 and 40 E1A proteins using the yeast import assay (Fig. 1C). Expression of the HAdV-5 E1A C-terminus (residues 187–289), which contains the canonical NLS, or just the NLS (residues 282–289) was sufficient to direct nuclear import, although at a reduced level as compared to the full length HAdV-5 E1A. Similarly, the C-terminal fragments from types representative of each other species induced nuclear import as well, or better than HAdV-5 C-terminus (Fig. 1C). Interestingly, the C-terminal fragment of HAdV-3 E1A displayed roughly double the import activity of the HAdV-5 E1A fragment, despite the fact that the RKLPR sequence it contains does not fit that of a canonical NLS. Deletion of the RKLPR sequence (HAdV-3 ΔNLS) greatly reduces import activity conferred by the HAdV-3 C-terminal fragment, suggesting that it does indeed function as an NLS. These results demonstrate that a common feature of all the E1A proteins tested is the presence of a functional canonical, or near canonical, NLS located at their C-terminus.

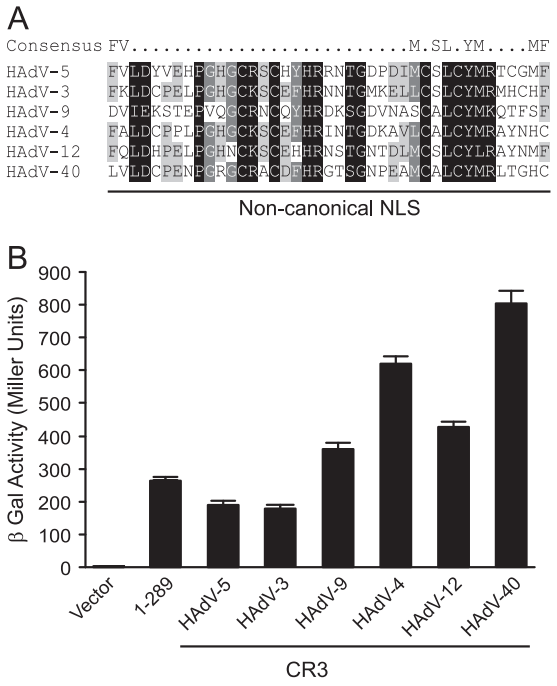

Fig. 2. Analysis of nuclear import mediated by conserved region 3 (CR3) of E1A from representative HAdV species. (A) Alignment of the putative non-canonical NLS sequences of E1A CR3 from representative HAdV types used in this study (Avvakumov et al., 2004). (B) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for CR3 E1A fragments from the indicated HAdV types (means ± SD, $n=3$). Vector—pNIA-CEN-MBP.

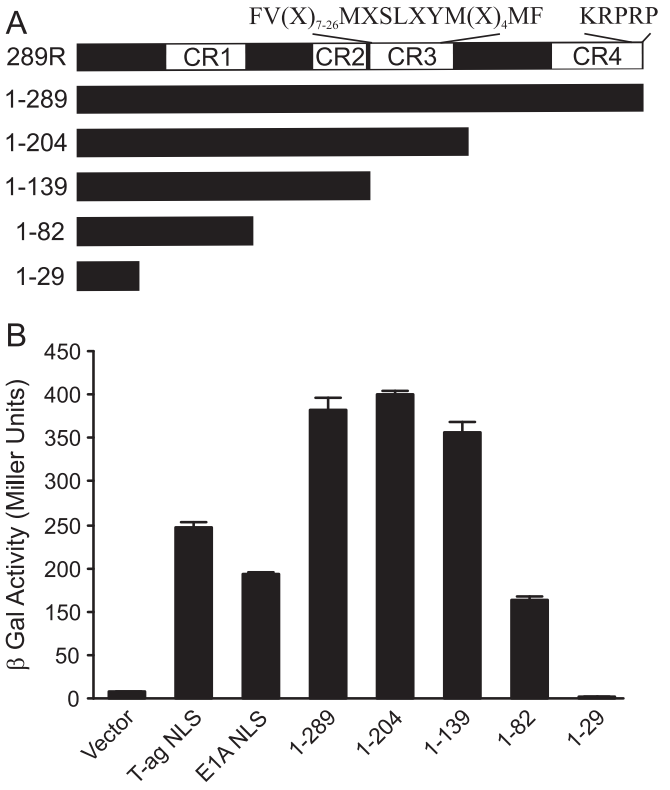

Fig. 3. Identification of a novel non-conventional nuclear localization signal (NLS) within the N-terminal/CR1 portion of HAdV 5 E1A. (A) Map of the 289R HAdV-5 E1A protein with previously identified regions known to confer nuclear import and the mutant constructs used in this study. (B) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for the C-terminal truncations of HAdV5 E1A. (means ± SD, $n=3$). T-ag NLS—SV40 Large T Antigen NLS, vector—pNIA-CEN-MBP.

The non-canonical NLS in E1A CR3 is functional in all HAdV species

A non-conventional NLS was identified within the CR3 of the HAdV-5 E1A protein, with the consensus sequence $FV(X)_{7-26}$-$MXSLXYM(X)_4MF$ (Standiford and Richter, 1992). Interestingly, none of the E1A proteins from the other HAdV species contain this consensus sequence (Fig. 2A). To determine if each of these regions could direct nuclear import, we tested the CR3 portions of HAdV-3, 4, 5, 9, 12 and 40 E1A using the yeast import assay (Fig. 2B). Expression of HAdV-5 E1A CR3 (residues 139–204), which contains this identified consensus sequence, was sufficient to induce nuclear import in yeast. The corresponding CR3 portions of E1A proteins representative of each HAdV species induced nuclear import more efficiently than HAdV-5 CR3 (Fig. 2B). These observations suggest that another common feature of all the E1A proteins tested is the presence of a functional non-canonical NLS within CR3, which does not follow the proposed consensus sequence.

A non-conventional NLS is located in the N-terminus of representative E1A proteins from each HAdV species and maps to residues 30–69 within HAdV-5 E1A

Previous experiments have indicated that the N-terminal region of HAdV-5 E1A contains a nuclear import activity (Richter et al., 1985). We constructed and tested a series of C-terminal truncations of HAdV-5 E1A for nuclear import activity in the yeast system to determine the location of this import signal (Fig. 3A). Truncation of the 289R E1A protein after residue 204 remove the canonical NLS located at the C-terminus. However, import activity was still observed, perhaps due to the non-conventional import signal located within CR3 (Fig. 3B). Truncation after residue 139 still maintained nuclear import activity, despite the loss of the non-conventional NLS within CR3 and the canonical NLS located at the C-terminus. A third fragment truncated following residue 82 maintained half the nuclear import activity seen with the 289R E1A protein, however truncation after residue 29 resulted in a loss of import activity. These results show that there is an additional novel non-conventional NLS located within the N-terminus/CR1 of E1A, specifically within residues 30–82 of HAdV-5 E1A. However, this signal functions less effectively than the canonical E1A NLS located at the C-terminus or the SV40 T-antigen (T-ag) NLS in this assay (Fig. 3B).

We next determined whether the NLS located within the N-terminal/CR1 portion of HAdV-5 E1A was present in the E1A proteins of all six HAdV species. The import activities mediated by the corresponding N-terminal portions of E1A from the other representative types were similar to that of HAdV-5 E1A, with the exception of HAdV-40 E1A, which was much higher (Fig. 4B). Interestingly, HAdV-40 E1A lacks the putative nuclear export signal (NES) (Fig. 4A), which is located between residues 70–80 in the N-terminal/CR1 portion of HAdV-5 E1A (Jiang et al., 2006). Therefore, the high levels of import activity seen with the N-terminal/CR1 portion of HAdV-40 E1A could result from enhanced accumulation of this fusion within the nucleus, due to its inability to be exported to the cytoplasm.

We also tested a panel of small deletions within the first 82 amino acid residues of HAdV-5 E1A to identify the specific region containing the import signal (Fig. 5A). As seen in Fig. 5B, deletion of residues 26–60 within the N-terminal/CR1 fragment completely abrogates import activity. Deletion of residues 70–81 (containing the previously described NES) increases import activity when compared to the wild-type 1–82 E1A fragment, suggesting the NES does indeed function in the yeast system. We also tested fragments of the N-terminus of E1A corresponding to residues 1–29, 30–69 and 70–82. Of these fragments, only residues 30–69

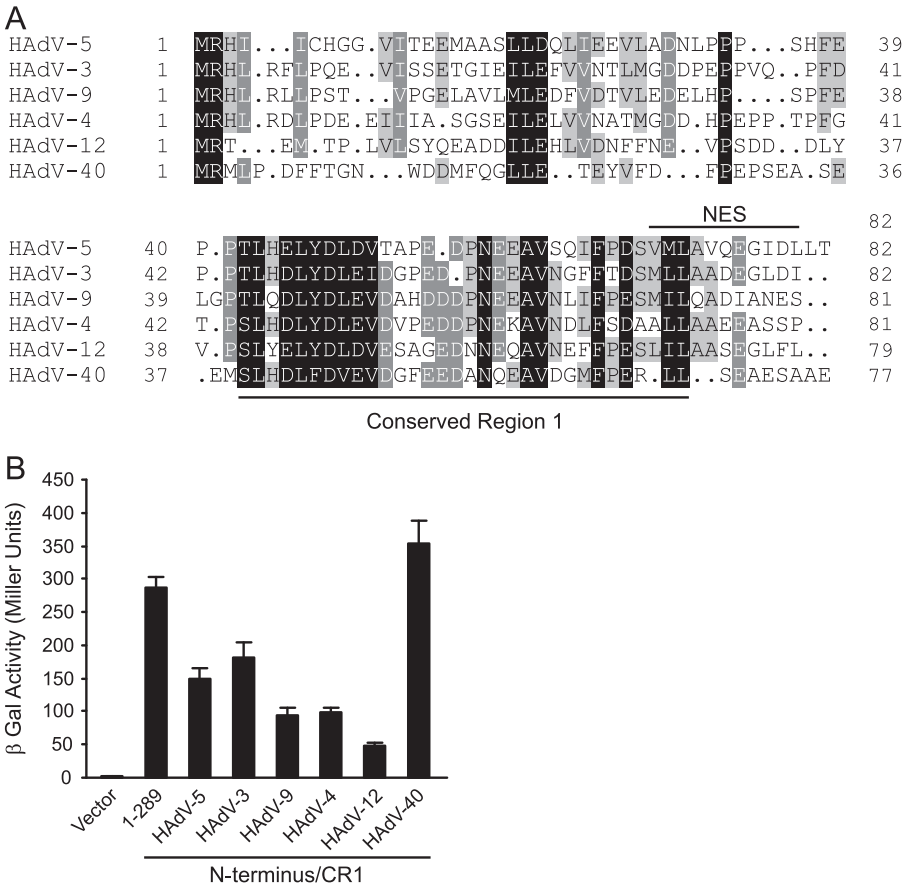

Fig. 4. Analysis of nuclear import mediated by the N-terminus of E1A from representative HAdV species. (A) Alignment of the N-terminal/CR1 E1A sequences from representative HAdV species. The position of the putative nuclear export signal (NES) is indicated. (B) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for the N-terminal/CR1 portions of E1A from the indicated HAdV types (means ± SD, $n=3$). Vector—pNIA-CEN-MBP.

was sufficient to direct nuclear import, which was comparable to full length 289R HAdV-5 E1A (Fig. 5B). These results show that the non-conventional NLS within the N-terminal/CR1 portion of HAdV-5 E1A is located within residues 30–69. Notably, this region of E1A contains no basic amino acids whatsoever, which are considered hallmarks of a conventional NLS.

The novel non-conventional NLS within the N-terminus of E1A interacts with yeast importin α

To determine if the non-conventional NLS within the N-terminal/CR1 portion of E1A functions via the classical nuclear import mechanism, we tested its ability to bind Srp1, the sole importin α in yeast. The interaction of HAdV-5 E1A and the E1A N-terminal deletion mutants with Srp1 was measured using the yeast two-hybrid assay (Fig. 5C). The observed interaction profile for Srp1 with E1A and the small deletion mutants was similar to the nuclear import activity seen in Fig. 5B. Importantly, residues 30–69 of HAdV-5 E1A also interact with Srp1 in this assay.

Nuclear localization directed by the N-terminal region of E1A functions through several adjacent subdomains

To further map the sequence requirements of the 30–69 region for nuclear localization, various truncations were constructed and tested in the yeast transcription based import assay (Fig. 6). Interestingly, all truncations retained some ability to confer nuclear import. However, all exhibited reduced activity, suggesting that the combined activities of several different subdomains confers strong nuclear import in these experiments.

The N-terminus of E1A can direct nuclear import in mammalian cells and interacts indirectly with mammalian importin α

In addition to our studies in yeast, we tested whether the N-terminus of E1A could similarly function to mediate nuclear import in mammalian systems. HeLa cells were transfected with a vector expressing GFP or GFP fused to various parts of E1A and the GFP signal was visualized by confocal microscopy (Fig. 7). Fusion with full length 289R E1A or the C-terminus of E1A quantitatively conferred nuclear localization. Fusion with the N-terminal region of E1A also stimulated nuclear localization, but significant fluorescence was also present in the cytoplasm (Fig. 7).

We also used the inducible translocation trap (ITT) mammalian nuclear import assay to test whether the N-terminus of E1A could direct nuclear localization in mammalian U2-OS cells (Saint and Fujii, 2008; Hoshino et al., 2004). This assay functions similarly to the yeast transcription based system, except that the LexA fusion is expressed in mammalian cells and activates luciferase expression if present in the nucleus. In this system, full length 289R E1A protein was able to induce import into the nucleus as measured by the increased luciferase activity with respect an otherwise identical LexA fusion lacking a functional NLS (Fig. 8A). As well, the C-terminal portion of E1A and CR3, which contain well studied NLSs, were also able to induce translocation into the nucleus as detected using this system (Fig. 8A). The N-terminal 82 residues of E1A also showed nuclear import activity in this mammalian assay, despite being weakly expressed as compared to the other tested constructs (Fig. 8A, lower). Residues 30–69 of HAdV-5 E1A were also sufficient to direct nuclear import of the fusion construct in this assay (Fig. 8A).

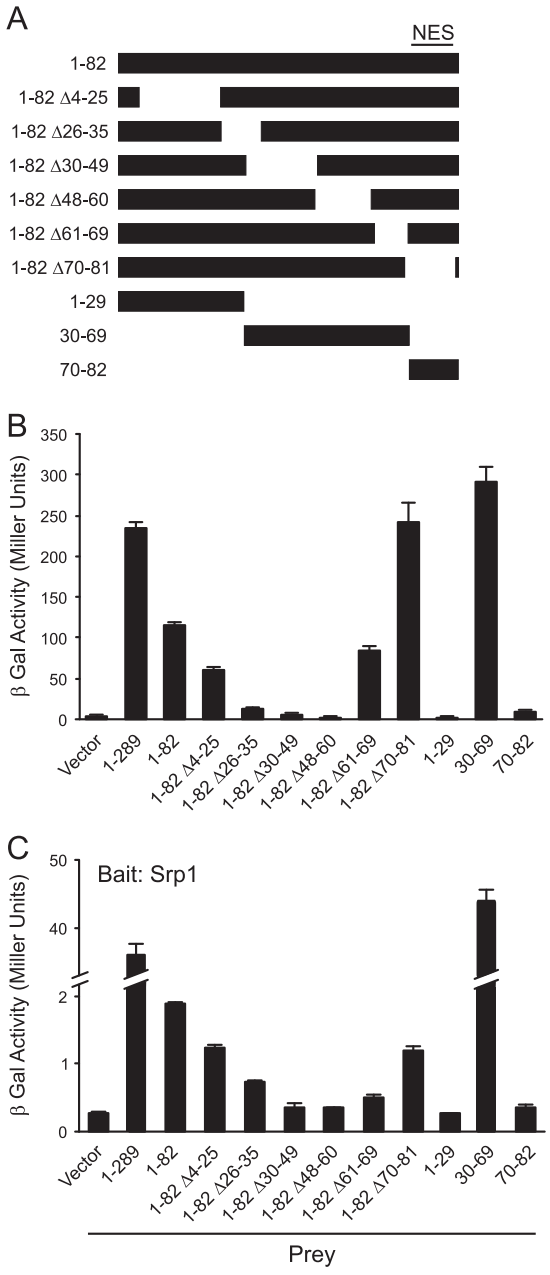

Fig. 5. Mapping the NLS within the N-terminus of HAdV 5 E1A. (A) Map of the HAdV-5 E1A N-terminus and the deletion mutants used in these experiments. (B) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for the indicated N-terminal/CR1 portions of E1A from HAdV-5 (means ± SD, $n=3$). Vector—pNIA-CEN-MBP. (C) The N-terminus of E1A interacts with yeast importin α, Srp1. Absolute values of ß-galactosidase reporter activity indicating interaction between the N-terminal/CR1 E1A fragments from the representative HAdV types and the yeast importin α, Srp1 (means ± SD, $n=3$).

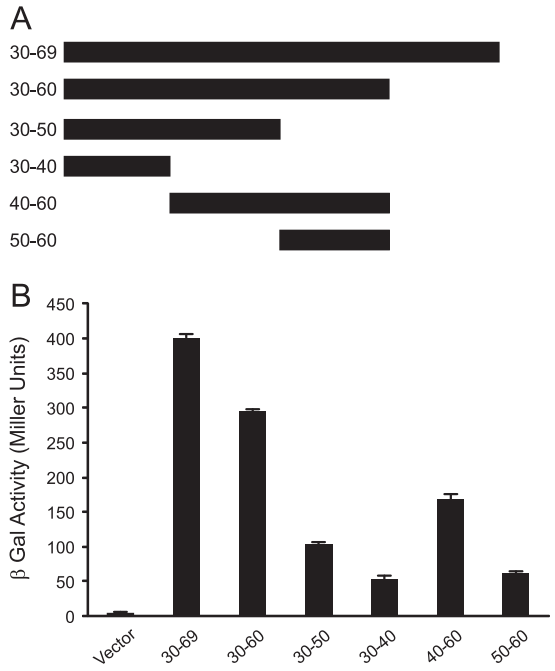

Fig. 6. Multiple sub-regions within the N-terminus of HAdV 5 E1A contribute to nuclear localization. (A) Map of the fragments of the HAdV-5 E1A N-terminus used in these experiments. (B) Absolute values of ß-galactosidase reporter activity indicating nuclear import in yeast for the indicated N-terminal fragments of E1A from HAdV-5 (means ± SD, $n=3$). Vector—pNIA-CEN-MBP.

Co-immunoprecipitation assays were also used to detect stable interactions between the N-terminal/CR1 portion of HAdV-5 E1A and the three subtypes of human importin α proteins: Rch1, Qip1 or NPI1 (Fig. 8B). Each of the human importin α proteins stably interacted with both the full length 289R E1A protein as well as the C-terminal portion of HAdV-5 E1A. Rch1 and Qip1, but not NPI1, also interacted with the N-terminal 82 residues of HAdV-5 E1A (Fig. 8B). This data suggests that this non-canonical NLS interacts selectively with specific importin α types.

To determine if the detected interaction between the N-terminal region and importin α was direct or indirect, we determined if purified recombinant fragments of E1A fused to GST could interact with recombinant Qip1 (Fig. 9). GST fusions of either full length 289R E1A or the C-terminal region containing the canonical NLS could interact directly with recombinant Qip1. No interaction was observed between Qip1 and either E1A residues 1–82 or 30–69, indicating that they do not directly interact with Qip1.

Discussion

The transport of viral proteins into the nucleus must overcome the barrier imposed by the nuclear envelope. Viral proteins have co-evolved with, and exploited the eukaryotic nuclear transport apparatus to overcome this barrier and gain entrance into the nucleus. HAdV E1A has apparently developed multiple mechanisms to navigate the nuclear pore and facilitate its role in the viral lifecycle. The HAdV-5 E1A gene is the first to be transcribed upon infection and the proteins it encodes are essential for viral replication, mainly through their ability to interact with, alter and utilize key cellular proteins involved in transcription (Frisch and Mymryk, 2002; Berk, 2005; Pelka et al., 2008). The majority of interactions that E1A makes with host cellular proteins occur within the nucleus and E1A has evolved to exploit the host nuclear transport mechanism to promote viral infection.

HAdV E1A proteins contain a predicted classical monopartite NLS located at their extreme C-terminus, with the exception of certain types from species B, including HAdV-3 (Avvakumov et al., 2004). Using a transcription based yeast nuclear import assay, we show that the E1A proteins from all six species, including HAdV-3 E1A, contain a functional NLS signal at this location (Fig. 1C). The sequence of the C-terminus of HAdV-3 E1A resembles a canonical monopartite NLS, as it is rich in basic amino acids and proline, yet does not match the normal consensus patterns used to identify such sequences (Nakai and Horton, 1999). These results indicate that a canonical, or near canonical monopartite NLS is a common feature of all HAdV E1A proteins, including those produced by the species B viruses.

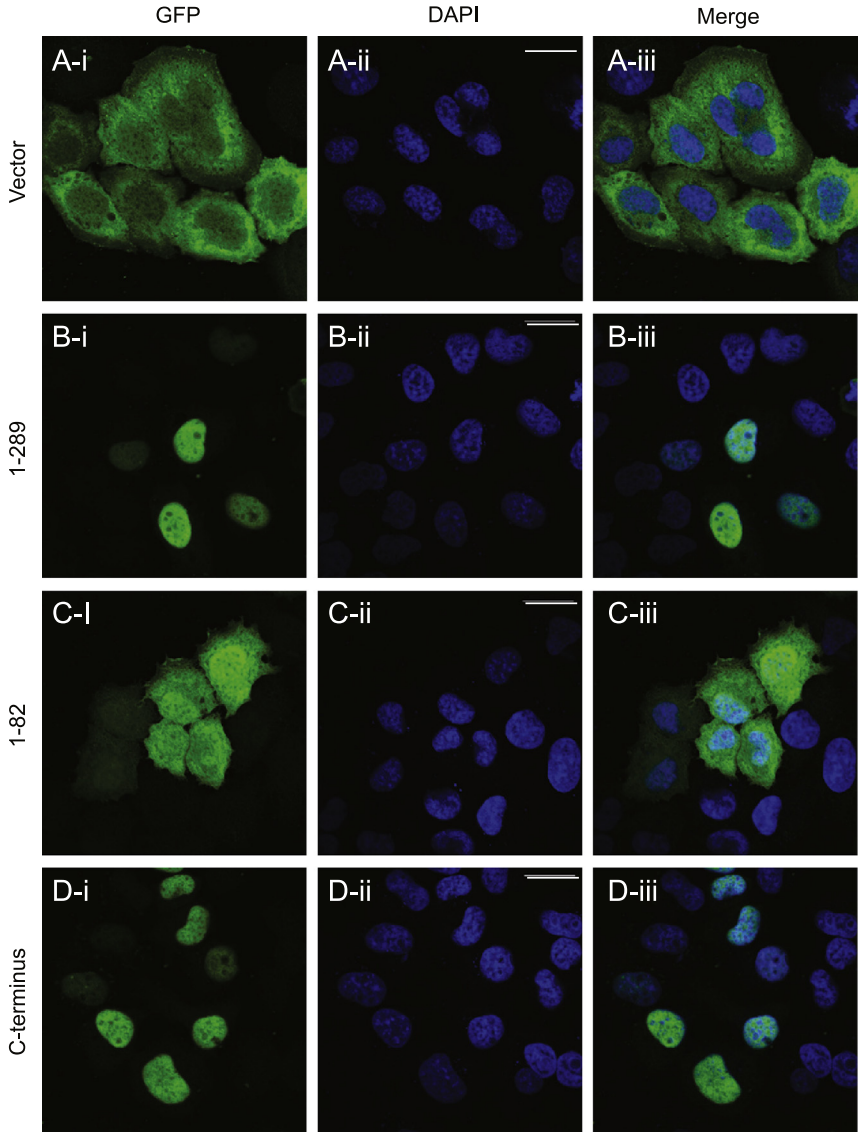

Fig. 7. The N-terminus of E1A directs import of GFP into the nucleus of mammalian cells. Human HeLa cervical carcinoma cells were transfected with expression vectors for GFP-fusions of full-length (a.a. 1–289), the N-terminus (a.a. 1–82), or the C-terminus (a.a. 187–289) of HAdV5 E1A and were subjected to confocal fluorescence microscopy. Nuclei were stained with DAPI. White scale bars represent 20 μm.

Despite the apparent universal presence of a C-terminal monopartite NLS in E1A, a second NLS has been reported in HAdV-5 E1A. This developmentally regulated non-conventional NLS is located within CR3 of the 289R HAdV-5 E1A protein and was previously identified using amino acid deletion and substitution analysis in *Xenopus* oocytes and embryos, with the consensus sequence $FV(X)_{7-26}MXSLXYM(X)_4MF$ (Slavicek et al., 1989; Standiford and Richter, 1992). This signal is not conserved in the E1A proteins of any of the other HAdV species (Fig. 2A). Nuclear import mediated by the non-conventional NLS within CR3 of HAdV-5 E1A, and the other five types, was readily detectable using the yeast import assay (Fig. 2B). These results indicate that a non-canonical NLS is a common feature of the CR3 portions of all HAdV E1A proteins, and that further refinement of the consensus sequence is required.

We further identified another non-canonical NLS within the N-terminal/CR1 portion of E1A that localized between residues 30 and 60 in HAdV-5 E1A (Fig. 5B). This region does not contain any of the basic amino acid residues typically considered hallmarks of a canonical NLS (Fig. 4A). This N-terminal/CR1 NLS activity was also universally present in the E1A proteins of all the different species (Fig. 4B). Interestingly, a mutant E1A lacking residues 2–85 was previously shown to localize only to the cytoplasm in infected BRK cells (Quinlan et al., 1988). This provides additional evidence to support our identification of a non-canonical NLS in the N-terminus of E1A and also suggests that this NLS was critical for nuclear import under those conditions in that cell type.

In these experiments, the N-terminal portion of HAdV-40 E1A showed an increased level of import activity when compared to other species (Fig. 4B). This may be explained by the absence of an NES in the CR1 portion of HAdV-40 E1A (Jiang et al., 2006), which would increase its effective concentration in the nucleus. Similarly, deletion of the NES from the 1–82 portion of HAdV-5 E1A (1–82 Δ70–81) also enhanced nuclear import (Fig. 5B). These results suggest that the NES is a common feature of all the representative types, with the exception of HAdV-40. Thus, the presence of both NLS and NES functions indicates that shuttling of E1A into and out of the nucleus is a common feature of most, but not all HAdV types. It is possible that one function of E1A may be to transport some or all of its associated protein into different cellular compartments. In support of this concept, interaction of E1A with Nek9 has been shown to diminish the amount of this kinase in the nucleus (Pelka et al., 2007).

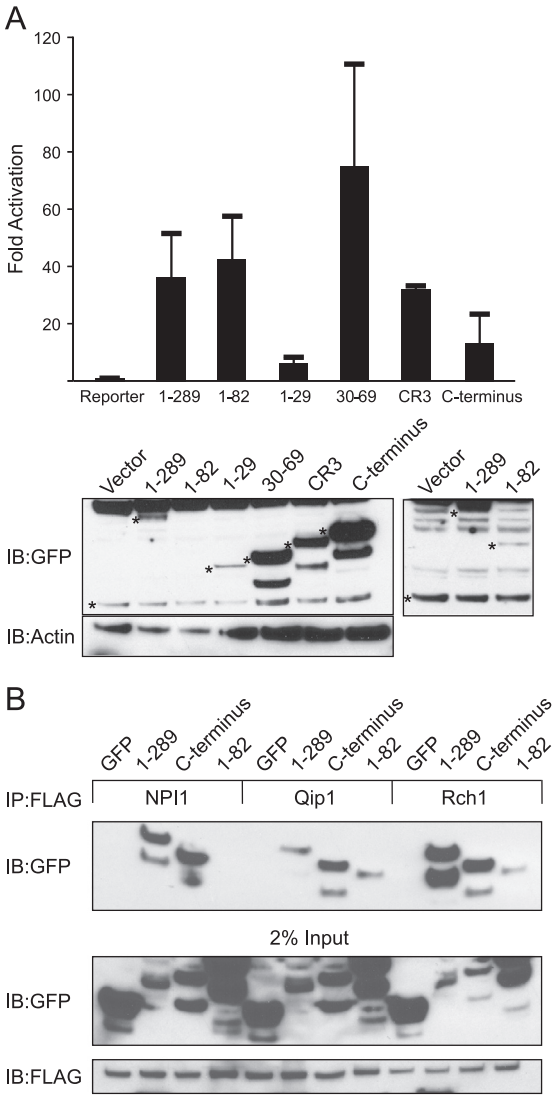

Fig. 8. The N-terminus of E1A directs import into the nucleus of mammalian cells and binds mammalian importins. (A) Several E1A segments can direct nuclear import. ITT assays were performed in U2-OS human osteosarcoma cells. Full length E1A and the indicated fragments of E1A were expressed using the pLGV vector along with a LexA responsive luciferase reporter as described in Materials and methods section. Luciferase activity was measured and results were normalized to protein (means ± SD, $n=3$). Western blot indicates relative level of expression of each fusion. The image on the right is a darker exposure of image on the left, showing expression of the 1–82 E1A fusion. Asterisks indicate the positions of each fusion. (B) Co-immunoprecipitation assays detect interactions between HAdV-5 E1A and representative mammalian importin α proteins. GFP fused to full length HAdV-5 E1A (1–289), the C-terminus of E1A or the N-terminus (1–82) of E1A were expressed in HT1080 cells along with the indicated FLAG tagged mammalian importin α proteins. Co-immunoprecipitations were performed using FLAG agarose. Western blot analysis was performed with the indicated antibodies. Representative images are shown. (means ± SD, $n=3$).

Non-conventional NLSs have been identified in other viral proteins, which interact with importin α to coordinate their nuclear import via the standard importin α/β pathway (Lischka et al., 2003; Cros et al., 2005). Typically, distinct importin α isoforms show differential interactions with specific cargo proteins. For this reason, different representative importin α proteins were tested for interaction with the non-conventional NLS within the N-terminal/CR1 portion of HAdV E1A. We initially tested the N-terminal/CR1 region of HAdV-5 E1A and a panel of deletion mutants for their interaction with Srp1, the single importin α in yeast. Two-hybrid analysis readily detected an interaction between yeast Srp1 and the N-terminal/CR1 portion of HAdV-5

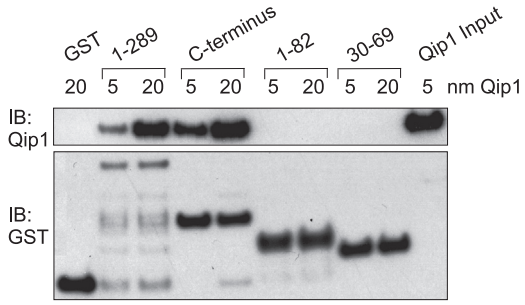

Fig. 9. The canonical C-terminal NLS, but not the non-canonical N-terminal NLS of E1A binds importin α directly. GST pulldowns were performed using the indicated portions of E1A fused to GST and recombinant Qip1. Blots were probed with anti-Qip1 antibody and reprobed with anti-GST antibody to confirm the presence the individual GST-E1A fusions.

E1A. Furthermore, the ability of the individual deletion mutants to bind Srp1 correlated with their ability to mediate nuclear import (c.f. Fig. 5B and C).

To translate these findings into a mammalian context, mammalian nuclear import assays were performed using the ITT assay. Similar to yeast assays, mammalian import assays showed substantial nuclear import activity by both of the well document nuclear localization signals within CR3 and the C-terminus of E1A (Fig. 8A). As well, the N-terminus showed nuclear import activity in human cells (Fig. 8A) similarly to the yeast nuclear import assays (Fig. 5B). Furthermore, confocal microscopy confirmed that fusion of residues 1–82 of E1A to GFP could direct nuclear localization of the chimeric protein in mammalian cells (Fig. 7). The similarity between the results obtained in the yeast and mammalian assays suggests a high level of evolutionary conservation and confirms the value of yeast as a model organism for the study of nuclear import. In addition, co-immunoprecipitation assays detected a physical interaction between importin α proteins and the HAdV-5 E1A N-terminal/CR1 region (Fig. 5C and Fig. 8B). These studies revealed that all mammalian importin α isoforms interact with the 289R HAdV-5 E1A protein and the C-terminus of E1A (Fig. 8B). In contrast, the N-terminal 82 residues of HAdV-5 E1A only interacted with Rch1 and Qip1. Thus, the different portions of E1A show specificity for different importin α proteins. We further determined that the N-terminus of E1A associates indirectly with Qip1, as these two purified recombinant proteins did not interact *in vitro* (Fig. 9). Thus, the ability of the N-terminus to direct nuclear localization appears to require a bridging factor, or factors, which themselves interact with importins, to allow cotransport into the nucleus via a "piggybacking" mechanism (Macara, 2001). Notably, the N-terminus of E1A functions as a strong transcriptional activator when fused to a DNA-binding domain (Yousef et al., 2009). This activity is mediated by the interaction of E1A with multiple transcriptional regulators (Pelka et al., 2008), and it is likely that it is these associations that direct cotransport of E1A into the nucleus.

Our findings confirm that, despite substantial differences in primary sequence, HAdV E1A proteins are remarkably consistent in that they contain one canonical and two non-canonical NLSs. The conservation of these additional nuclear import signals present in each of the various types is highly suggestive that under some conditions the C-terminal NLS is not sufficient to localize E1A to the nucleus. Although these exact conditions have not been conclusively identified, it is known that acetylation of the C-terminal NLS blocks association with importin α (Madison et al., 2002). Given that the C-terminal canonical NLS utilizes the all importin α proteins including Qip1 (Kohler et al., 2001), whereas the non-canonical NLS in the N-terminal/CR1 region

appears to preferentially target Rch1 and Qip1, it seems likely that E1A exploits multiple mechanisms to ensure nuclear localization in the infected cell regardless of the cellular repertoire of importin α expression.

Materials and methods

Plasmid construction

The construction of pNIA-CEN-MBP and derivatives expressing the SV40 large T antigen NLS, the 289R E1A protein, residues 1–82, 139–204, 187–289 or the canonical C-terminal NLS of HAdV-5 E1A was described previously (Marshall et al., 2007). This low copy yeast plasmid expresses a modified LexA DNA binding domain fused to the *Escherichia coli* maltose binding protein (MBP), the Gal4 transcriptional activation domain and an HA epitope tag from the *ADH1* promoter. Even without the addition of a test sequence, this >80 kDa fusion protein is substantially larger than the diffusion limit of the yeast nuclear pore complex (Shulga et al., 2000) and is excluded from the nucleus (Marshall et al., 2007). All other constructs were generated by PCR and inserted into pNIA-CEN-MBP or subcloned from other plasmids (Shuen et al., 2002, 2003; Avvakumov et al., 2002). Similar fusions were constructed using the vector pLGV, which is a mammalian expression vector encoding the LexA DNA binding domain fused to EGFP and the VP16 transcriptional activation domain (Hoshino et al., 2004). E1A fusions with EFGP or GST were previously described (Pelka et al., 2011; Shuen et al., 2002). Transcriptional activation domain fusions of various portions of the E1A proteins were constructed by PCR and inserted into the yeast two-hybrid prey vector pJG4-5 (OriGene Technologies Inc., Rockville, MD). SRP1 was amplified by PCR and subcloned into pBAIT (Zhang et al., 2001). The human importin α genes were subcloned into a pCDNA3 vector with FLAG and HA tags from plasmids provided by Dr. Hiroshi Masutani.

Yeast nuclear import and two-hybrid β-galactosidase assays

Yeast transformations for the nuclear import and two-hybrid β-galactosidase assays were performed as described previously (Marshall et al., 2007). All results shown represent the average of three replicates.

Mammalian nuclear import assay

Human U2-OS osteosarcoma cells were transfected with the pLGV vector (Hoshino et al., 2004) or derivatives expressing either full length HAdV-5 E1A or the indicated fragments and a LexA-responsive luciferase reporter gene (Lew and Elsholtz, 1995). After 48 h, cells were lysed in 150 μL using the supplied lysis buffer (Promega E397A). For detection of luciferase production, 50 μL of lysate was mixed with 50 μL of Luciferase Substrate (Promega E151A) immediately before detection of light as measured using a Berthold Lumat LB 9507. Results were then normalized to protein levels and presented as fold increase over reporter and pLGV alone.

Immunofluorescence microscopy

Cells were fixed on glass coverslips in 3.7% paraformaldehyde, permeabilized on ice using 0.2% Triton X-100 and blocked using 5% goat serum in PBS. Samples were incubated with primary antibody (Rabbit anti-GFP, Clontech) at room temperature for 1 h and for another hour at room temperature with secondary antibody (AlexaFluor-488 anti-Rabbit, Life Technologies). Coverslips were mounted on glass microscope slides using ProLong Gold Anti-Fade reagent with DAPI (Life Technologies). Confocal images were acquired with a Fluoview 1000 laser scanning confocal microscope (Olympus Corp).

Co-immunoprecipitation and Western blot analysis

Human HT1080 fibrosarcoma cells were transfected with vectors expressing the indicated mamalian importin α and E1A or E1A segment. After 24 h, cells were lysed with NP40 lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.1% NP-40) and protein concentrations were determined with BioRad protein assay reagent using BSA as a standard. Lysate was immunoprecipitated with FLAG agarose at 4 °C for 4 h. 2% input of protein was kept as a loading control. Nitrocellulose membranes were then probed with an anti-FLAG or anti EGFP antibodies.

Recombinant protein purification and GST-pulldown assays

Recombinant GST-tagged E1A and Qip1 proteins were purified from the BL21-RIL strain of *E. coli* per protocols provided by the affinity resin manufacturer (Bio Basic Canada Inc.). To obtain untagged Qip1, purified recombinant GST-tagged Qip1 was incubated with ~0.2 μg/mL TEV protease overnight at 4 °C. The ability of Qip1 to associate with full-length E1A or indicated fragments was assessed *in vitro* using a GST-pulldown assay. Briefly, 20 nM of GST or GST-E1A was incubated with 5 or 20 nM of purified Qip1 at 4 °C for 2 h in a total volume of 200 μL of buffer (20 mM HEPES pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, 50 μg/mL BSA) and 20 μL of 50% glutathione Sepharose. Samples were washed three times with phosphate buffer, boiled in 2× LDS sample buffer for 5 min, then separated by SDS-PAGE and examined by Western blotting with antibodies again GST (Sigma) or Qip1 (Thermo Fisher Scientific).

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